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(72) Inventors: GUDKOV, Andrei, V.; 700 Sunset Lane, Glencoe, IL 60022 (US). KOMAROVA, Elena, A.; 1024 Home Avenue, Oak Park, IL 60304 (US).

(54) Title: P53–DEPENDENT SECRETION OF GROWTH INHIBITORY FACTORS

(57) Abstract

    The present invention relates to a method for preparing a preparation of p53–dependent growth inhibitory factors for use in a pharmaceutical composition. Cultured human cells, capable of expressing p53 tumor suppressor protein, are induced to express and secrete p53–dependent growth inhibitory factors into the culture medium, where the growth inhibitory factors are recovered to prepare a preparation for use in a pharmaceutical composition. This pharmaceutical composition is then used in a method for reducing unwanted or abnormal cell proliferation by administering an effective amount to a subject in need thereof.

P53-DEPENDENT SECRETION OF GROWTH INHIBITORY FACTORS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority under 35
5 U.S.C. §119(e) from U.S. Provisional application nos.
60/075,490, filed February 23, 1998, and 60/084,755, filed May
8, 1998, the entire contents of which are hereby incorporated
by reference.

BACKGROUND OF THE INVENTION

10 Field of the Invention

The present invention relates generally to the p53-
dependent secretion of growth inhibitory factors and more
particularly to a method for preparing p53-dependent growth
inhibitory factors for use in a pharmaceutical composition and
15 a method for reducing cell proliferation.

Description of the Related Art

The p53 tumor suppressor gene encodes a nuclear
transcription factor (tumor suppressor protein), that
20 accumulates in the cells in response to a variety of stresses
and that is a key component of a cellular mechanism of stress
response (for review see Gottlieb et al., 1996). This tumor
suppressor protein, which has rapid turnover, is stabilized in
the cell under various stressful conditions including DNA
25 damage (Maltzman et al., 1984; Kastan et al., 1991),
deregulation of microtubules (Tishler et al., 1995), hypoxia
(Graeber et al., 1996) and detachment from its natural
substrate (Nikiforov et al.). Normal function of p53 is
required for control of cell aging (Atadja et al., 1995).
30 Cell response to certain growth factors also requires p53
(Bacus et al., 1996). p53 activation, depending on cell type,
results in growth arrest at one of the cell cycle check points
or apoptosis (Michalovitz et al., 1990).

The biological effects of p53 are cell type
35 specific. For example, in vivo it determines massive
apoptosis in response to gamma irradiation or treatment with

chemotherapeutic drugs only in early embryos and several
sensitive tissues, including spleen and thymus (Lowe et al.,
1993a; MacCallum et al., 1996; Komarova et al., 1997). Many
p53-mediated effects are achieved through the activity of p53-
5 responsive genes.

        Functional inactivation of p53 occurs in the
majority of cancers and established cell lines (Vogelstein et
al., 1992). It is achieved by mutations (many of which
generate dominant negative variants of p53), deletions or
10 inhibitory activity of p53-interacting proteins of cellular
(such as mdm-2) or viral (such as large SV40 T-antigen, E6
protein or papilloma virus, etc.) origin (see references cited
in Gottlieb et al., 1996). Lack of p53 in normal cells leads
to the loss of genomic stability (Yin et al., 1992),
15 spontaneous immortalization (Donehower et al., 1992),
abrogation of apoptosis (Clarke et al., 1993 and Lowe et al.,
1993a) and as a result, susceptibility to transformation with
dominant oncogenes (Olson et al., 1994). Loss of p53 by tumor
cells may be associated with high resistance to chemo- and
20 radiation therapy and the establishment of metastatic behavior
through suppression of p53-mediated programmed cell death
(Nikiforov et al.; Lowe et al. 1993a; Lowe et al., 1993b and
Thompson et al., 1995). In normal development, p53 serves to
control apoptosis elimination of genetically damaged cells in
25 early stages of embroyogenesis (Norimura et al., 1996 and
Komarova et al., 1997). Lack of p53 in p53 knock-out mice
leads to a high incidence of spontaneous cancers in adult
animals (Donehower et al., 1992; Jacks et al., 1994) and to
high frequency of teratogenic abnormalities in embryos exposed
30 to ionizing radiation (Norimura et al., 1996). The exact
mechanisms by which p53 exerts its growth-suppressor and
apoptoxic activities are poorly understood, although some of
the components of the p53-medicated control pathway have been
identified (Gottlieb et al., 1996). At least some of the p53-
35 mediated effects are determined by the ability of p53 to act
as a nuclear transcription factor. The activity of p53-
responsive genes accounts in part for p53-mediated checkpoint

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control (*wafl; 14-3-3*), apoptosis (*bax*), genes determining
reactive oxygen metabolism, and p53 feedback regulation (*mdm2*)
(El-Deiry et al.,1993; Hermeking et al., 1997; Kastan et al.,
1992; Wu et al., 1993; Miyashita et al., 1995; Okamoro et al.,
5 1994; Buckbinder 1995; Polyak et al., 1997; Momand et al.,
1992 and Kondratov et al., 1995). In addition, p53 can
suppress transcription from a number of promoters (Mack et
al., 1993) and directly inhibit DNA replication (Cox et al.,
1995).

10   Anti-cancer therapy regimens involve a variety of
stress-inducing treatments (radiation, chemotherapeutic drugs,
hyperthermia, etc.) the toxicity of which is higher for
cancer cells than for normal cells. Many of these treatments
lead to the activation of p53 and subsequent p53-dependent
15 growth arrest or apotosis (Gottlieb et al., 1996). The
conventional mode of thinking is that cell death occurs as a
result of damage of intracellular components that trigger cell
death, and the anti-cancer effect is believed to be associated
either with stronger damage or with stronger apoptotic
20 response of malignant versus normal cells. Since anti-cancer
therapy is usually damaging not only to the tumor but also to
normal tissues, much effort has been made in targeting stress
treatment specifically and selectively at cancer cells.
However, there is a bulk of evidence indicating that anti-
25 cancer therapy with stress-inducing treatments is not limited
to the internal damage occurring within cancer cells after
therapeutic treatment. For example, shielding the tumor in a
tumor-bearing animal does not completely prevent it from
exhibiting the anti-tumor effect of whole-body gamma
30 irradiation (Seung et al., 1996). A decreased ability of
tumors to grow in irradiated tissues, known as the "tumor bed

effect", is a well-known phenomenon (Milas et al., 1988;
O'Brien et al., 1969; Urano 1967).
35   In response to treatment with radiation or
hyperthermia, or with DNA-damaging or microtubule-targeted
chemotherapeutic drugs, p53 protein accumulates in the cell

(Gottlieb et al., 1996). The tolerable doses of anticancer
drugs and radiation are limited by their toxicity to several
sensitive tissues, including hematopoietic organs, where
transgenic animal studies showed that this effect is p53
5 dependent (Komarova et al., 1997). Thus, the efficacies of
chemo- and radiation therapies are mediated by p53, and at the
same time, are limited by p53-dependent toxicity for normal
tissues.

Citation of any document herein is not intended as
10 an admission that such document is pertinent prior art, or
considered material to the patentability of any claim of the
present application. Any statement as to content or a date of
any document is based on the information available to
applicant at the time of filing and does not constitute an
15 admission as to the correctness of such a statement.

SUMMARY OF THE INVENTION

It is an object of the present invention to overcome
the deficiencies of the prior art.
20 The present invention firstly provides a method for
preparing a preparation of p53-dependent growth inhibitory
factors from a culture of normal human cells in a nutrient
medium for use as antiproliferative agents, such as an anti-
cancer therapeutic.
25 The present invention further provides a
pharmaceutical composition comprising a preparation of p53-
dependent growth inhibitory factors prepared from a culture of
human cells induced to express and secrete p53-dependent
growth inhibitory factors.
30 Also provided by the present invention is a method
for reducing unwanted or abnormal cell proliferation by
administering a pharmaceutical composition containing a
preparation of p53-dependent growth inhibitory factors.

Another object provided by the present invention is
35 a method for preventing or delaying cell senescence by
administering a pharmaceutical composition containing an agent
which attenuates the secretion of or neutralizes the growth

suppressive effects of one or more p53-dependent growth inhibitory factors.

BRIEF DESCRIPTION OF THE DRAWINGS

5   Figure 1 shows a graph of the results of a tumor growth inhibition assay, which demonstrates tumor suppression *in vivo* by conditioned medium. Mice were examined weekly for tumor growth. Four weeks after injection, tumors developed in approximately half of the injected sites in the control group,
10 while the experimental group of injected animal tumors appeared in only three of 20 sites injected. This result suggests that factors secreted by irradiated splenocytes possess *in vivo* detectable anticancer activity.

          Figures 2A and 2B show detection of p53-responsive
15 genes by differential hybridization with ATLAS cDNA Array (CLONTECH Laboratories, Inc., Palo Alto, CA) (Fig. 2A) and Northern blot hybridization (Fig. 2B). In Fig. 2A, similar areas of nylon membranes hybridized with ^{32}P-labeled cDNA from three organs (liver, spleen, and thymus) of
20 untreated and gamma-irradiated p53-null and wild type mice are shown. Radiation responses of three indicated serine protease inhibitors illustrate the tissue-specificity and p53-dependence of their expression. The results of hybridization to cDNA arrays were confirmed by Northern blot hybridization
25 for serine protease inhibitor 2 (Fig. 2B). The abbreviations shown in Figs. 2A and 2B are as follows: 1 - α1-protease inhibitor 2; 2- serine protease inhibitor 2; 3- serine proteinase inhibitor 2.4; -wt- wild type; -/- -p53 knockout; u/t - untreated; and γ - gamma irradiated.
30   Figures 3A and 3B show different patterns of p53-dependent gene regulation as determined by Northern hybridization with RNA from cells that have been treated as indicated - untreated (u/t) MDAHO41 cells gamma irradiated (γ) MDAHO41 cells, tetracycline added (+ tetr) TR9-7 cells, no
35 tetracycline added (- tetr) cells and gamma irradiation + tetracycline added (γ + tetr).

Figures 4A-4F show that growth inhibition of
prostate tumor cells DU145 by conditioned media and urine
depends on p53 activation and p53 induction (Figs. 4A-4C) and
p53 induction by Western immunoblotting (Figs. 4D-4F). Bar
5 diagrams (Figs. 4A-4C) show cell growth rate estimated as
percent of ^{3}H-thymidine incorporation by the DU145 cells,
growing in the presence of conditioned medium from untreated
(left bar of bar pairs, considered as 100%) and gamma-
irradiated (right bar of bar pairs) cells. Cells used as
10 sources of conditioned media: Fig 4A - mouse embryo
fibroblasts (MEF) transformed with Ela + ras, p53-deficient (-
/-), p53 wild type (wt), transduced with either bcl-2 (bcl-2)
or anti-p53 GSE (GSE); Fig. 4B - Balb 3T3 cells, line ConA
(wt) and their derivatives expressing anti-p53 GSE (GSE); and
15 Fig. 4C - human skin fibroblasts MDAHO41 (-/-) and TR9-7 (wt).
The hatched bar in Fig. 4C corresponds to human fibroblasts
(line TR9-7) with tetracycline-regulated wild type p53
activated by tetracycline deprivation. Figs. 4D-4F show the
results of Western immunoblotting with MEF (Fig. 4D) and human
20 fibroblasts (Fig. 4F), demonstrating p53 induction after gamma
irradiation, and X-gal staining of ConA cells (marked Balb
3T3) (Fig. 4E).

Figures 5A-5C shows the growth suppressive effect of
conditioned media from short-term cultures of irradiated p53-
25 null (p53 -/-) and wild type (wt) splenocytes (Fig. 5A)
collected at the different time points indicated and urine
(Fig. 5B) from p53-null and p53-wild type mice before
(control) and after gamma irradiation (gamma).
Immunoperoxidase staining of irradiated p53-wild type and p53-
30 null splenocytes from p53 is shown in the left bottom corner
of Fig. 5A. In the representative experiment shown, urine
samples from six p53-wild type (crosses) and six p53-null mice
(dots) harvested before and after irradiation were tested
individually (Fig. 5B). Fig. 5C shows a comparison of growth
35 suppressive effect of different dilutions of conditioned
medium from gamma irradiated ConA cells on different indicated
target cells (NMuMG, HSF, and DU145). The conditioned serum-

free medium was concentrated (eight fold) by ultrafiltration through Amicon filter with 10 kd cutoff.

Figures 6A-6D show adriamycin induced degradation of colonies of multidrug resistant KB/8-5-11 cells growing on
5 p53-wild type (Figs. 6A and 6C), but not on p53-null (Figs. 6B and 6D), human fibroblasts. Representative microscopic view at 10x (Figs. 6A and 6B) and 40x (Figs. 6C and 6D) magnification of colonies of KB8-5-11 cells after 5 days of treatment with 50 ng/ml of adriamycin are shown.
10 Figure 7 shows a graph of a comparison of adriamycin dependence on numbers of colonies formed by KB8-511 cells plated on wild type and p53-deficient human fibroblast.

DETAILED DESCRIPTION OF THE INVENTION

15 The present invention is based on the discovery by the present inventors that the activation of p53 in normal noncancerous cells induces the expression and secretion of growth inhibitory factors possessing anti-cancer activity. The present inventors found an accumulation of radiation-
20 induced factors, which cause antiproliferative effects on a variety of tumor cell lines, in the media from established and primary cell cultures and in vivo in the urine of mice after gamma irradiation in vitro. Moreover, it was found that p53-dependent factors released by normal cells potentiated the
25 cytotoxic effect of chemotherapeutic drugs for co-cultivated tumor cells. Accordingly, p53-dependent growth inhibitory factors secreted by normal cells play a role in the chemo- and radiation therapies for treating cancer.

One aspect of the present invention is directed to a
30 method for preparing a preparation of p53-dependent growth inhibitory factors from cultured normal human cells by subjecting the cell culture to a stress treatment, i.e., gamma irradiation, to induce the expression and secretion of p53-dependent growth inhibitory factors. The p53-dependent growth
35 inhibitory factors secreted into the culture medium to form a conditioned medium is then recovered to prepare a preparation of p53-dependent growth inhibitory factors for use in the

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second aspect of the invention, which is a pharmaceutical
composition for reducing unwanted or abnormal cell
proliferation.

This method for preparing a preparation of p53-
5 dependent growth inhibitory factors involves culturing human
cells so that p53 tumor suppressor protein is expressed in
response to a stress treatment (i.e., preferably gamma
irradiation), which p53 tumor suppressor protein acts to
induce the expression and secretion of p53-dependent growth
10 inhibitory factors into the culture medium. The conditioned
medium formed by the release of growth inhibitory factors into
the nutrient culture medium is then processed to recover the
p53-dependent growth inhibitory factors (i.e., TGF-beta 2,
inhibin-beta, and serine protease inhibitors) and provide a
15 preparation of such growth inhibitory factors.

The human cells used in the method according to the
present invention are preferably fibroblasts cells, spleen or
thymus cells, and mixtures thereof. Other suitable cells for
use in the present method can be readily identified by
20 determining whether they express p53 in response to a stress
treatment, the expression which of then induces the expression
and secretion of one or more p53-dependent growth inhibitory
factors. As the selection of p53-dependent growth inhibitory
factors expressed vary depending on cell type, a mixed culture
25 of different cell types can be used to produce a preparation
containing a broad spectrum of p53-dependent growth inhibitory
factors.

Many mammalian cell cultures are routinely grown in
nutrient culture media containing serum, where fetal bovine
30 serum (FBS) is probably the most extensively used serum for
mammalian cell culture, although other mammalian sera are also
used. However, the use of serum poses a number of problems.
Serum is an expensive commodity which is not readily available
in amounts required for commercial production. It is also a
35 biochemically undefined material. Serum is known to contain
many major components including albumin and transferrin and
also minor components, many of which have not been fully

identified nor their action determined. Thus, serum will
differ from batch to batch possibly requiring testing to
determine levels of the various components and their effect on
the cells. Frequently, serum is contaminated with
5 microorganisms such as viruses and mycoplasma, many of which
may be harmless but will represent an additional unknown
factor. This problem has become more acute in recent years
with the emergence of Bovine Spongiform Encephalopathy (BSE).
Despite improvements in screening, regulatory authorities are
10 likely to require the sourcing of bovine products from those
areas which are free from (BSE) infections. Furthermore, the
presence of animal proteins in culture media can require
lengthy purification procedures.

        While the conventional culture of human cells in a
15 nutrient culture medium containing serum can be used in the
method of the present invention, it is preferred that the
culture medium used for producing a preparation of p53-
dependent growth inhibitory factors be serum-free. Such
serum-free media, with or without specific substitutes for
20 components/factors in serum, is a defined media from which a
preparation of p53-dependent growth inhibitory factor can be
purified. Examples of such serum-free media suitable for
human cells are the commonly used DMEM and RPMI media are well
known. Many other serum-free media with or without
25 substitutes for serum component are also well known in the art
with some being commercially available from suppliers such as
Life Technologies, Gaithersburg, MD. Those of skill in the
art are furthermore quite capable of optimizing the defined
components of a serum-free media for the growth of the cell
30 type(s) to be used in the method according to the present
invention.

        As the serum-free media is preferably a defined
media, all the components of the media are known. Therefore,
one of skill in the art is quite capable of developing with
35 only routine experimentation a purification scheme for
removing media components to prepare a preparation of p53-
dependent growth inhibitory factors suitable for administering

to a subject in a pharmaceutical composition. Depending on
the components of the serum-free culture medium used in the
method of the present invention, repeated cycles of
ultrafiltration and washes may sufficiently remove the media
5 components that little or no further purification procedures
are needed to prepare the preparation of p53-dependent growth
inhibitory factors.

       The preparation of p53-dependent growth inhibitory
factors prepared according to the method of the present
10 invention can be formulated into a pharmaceutical composition
for use in reducing unwanted or abnormal cell proliferation.
An effective amount of the pharmaceutical composition is
administered to a subject in need thereof, such as a cancer
patient, in a method for reducing cell proliferation where the
15 proliferation of cells is unwanted or abnormal, i.e.,
neoplasms and malignant cancer cells. While the method for
reducing unwanted or abnormal cell proliferation may involve
the administration of the pharmaceutical composition
containing a preparation of p53-dependent growth inhibitory
20 factors as the sole treatment, it may be preferable and
advantageous to combine this administration with conventional
chemotherapy and/or radiation therapy to potentiate the
cytotoxic effects of chemotherapeutic drugs or enhance the
antiproliferative effects of radiation therapy.
25   The pharmaceutical composition of the present
invention is administered and dosed in accordance with good
medical practice, taking into account the clinical condition
of the individual patient, the site and method of
administration, scheduling of administration, patient age,
30 sex, body weight and other factors known to medical
practitioners. The pharmaceutically "effective amount" for
purposes herein is thus determined by such considerations as
are known in the art. The amount must be effective to achieve
improvement including but not limited to improved survival
35 rate or more rapid recovery, or improvement or elimination of
symptoms and other indicators as are selected as appropriate
measures by those skilled in the art.

In the method for reducing unwanted or abnormal cell proliferation according to the present invention, the compound of the present invention can be administered in various ways. It should be noted that the preparation of p53-dependent
5 growth inhibitory factors can be administered alone as the pharmaceutical composition or as active ingredients in combination with pharmaceutically acceptable carriers, diluents, excipients, adjuvants, vehicles and auxiliary agents. The pharmaceutical composition can be administered
10 orally, subcutaneously or parenterally including intravenous, intraarterial, intramuscular, intraperitoneally, and intranasal administration as well as intrathecal and infusion techniques. Implants containing the preparation are also useful. Moreover, it may be advantageous and preferable to
15 administer the pharmaceutical composition according to the present invention locally at or near the site of unwanted or abnormal cell proliferation, i.e., tumors. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles as well as implant carriers generally refer to inert,
20 non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention.

It is noted that humans are treated generally longer than the mice or other experimental animals exemplified herein
25 which treatment has a length proportional to the length of the disease process and drug effectiveness. The doses may be single doses or multiple doses over a period of several days, but single doses are preferred.

30   When administering the pharmaceutical composition of the present invention parenterally, it will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or
35 dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example,

water, ethanol, polyol (for example, glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils.

Proper fluidity can be maintained, for example, by
5 the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Nonaqueous vehicles such as cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl
10 myristate, may also be used as solvent systems for compound compositions. Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action
15 of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the
20 injectable pharmaceutical form can be brought about by the use of agents delaying absorption, to the present invention, however, any vehicle, diluent, or additive used would have to be compatible with the compounds.

Sterile injectable solutions can be prepared by
25 incorporating the preparation of p53-dependent growth inhibitory factors utilized in practicing the present invention in the required amount of the appropriate solvent with various of the other ingredients, as desired.

A pharmacological formulation of the present
30 invention can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicle, adjuvants, additives, and diluents; or the compounds utilized in the present invention can be administered parenterally to the patient in the form of slow-release
35 subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, and microspheres. Examples of

delivery systems useful in the present invention include:
5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678;
4,487,603; 4,486,194; 4,447,233; 4,447,224; 4,439,196; and
4,475,196. Many other such implants, delivery systems, and
5 modules are well known to those skilled in the art.

It is contemplated that a pharmacological
formulation of the compound utilized in the present invention
can be administered orally to the patient. Conventional
methods such as administering the compounds in tablets,
10 suspensions, solutions, emulsions, capsules, powders, syrups
and the like are usable. Known techniques which deliver it
orally or intravenously and retain the biological activity are
preferred.

In one embodiment, the pharmaceutical composition of
15 the present invention can be administered initially by
intravenous injection to bring blood levels to a suitable
level. The patient's levels are then maintained by an oral
dosage form, although other forms of administration, dependent
upon the patient's condition and as indicated above, can be
20 used. The quantity to be administered will vary for the
patient being treated and will vary from about 100 ng/kg of
body weight to mg/kg of body weight per day and preferably
will be from 10 μg/kg to 10 mg/kg per day.

The laboratory of the present inventors have also
25 discovered that the onset of senescence in rat and chicken
embryo fibroblasts is associated with the accumulation of
growth inhibitory factors in conditioned medium. Culture
media conditioned by senescent rat embryo fibroblasts (REFs)
was growth suppressive to a broad variety of target cells
30 including young REFs and a series of tumor cell lines. The
growth suppressive effect was neutralized after 1 hr.
incubation of conditioned media at 56°C. These observations
suggest that the aging of a cell population is a cooperative
process which involves growth suppression of proliferating
35 cells by growth inhibitors released by gradually accumulating
senescent cells.

Accordingly, a further aspect of the present

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invention is directed to a method for preventing or delaying
cell senescence, which involves administering to a subject in
need thereof a pharmaceutical composition containing an agent
which has the activity of directly or indirectly attenuating
5 the secretion of or neutralizing the growth suppressive
effects of p53-dependent growth inhibitory factors released by
gradually accumulating senescent cells. The features of
formulating the agent in a pharmaceutical composition, dosage
and how it is administered, as discussed above with the
10 pharmaceutical composition containing a preparation of p53-
dependent growth inhibitory factors, also apply to a
pharmaceutical composition containing this agent for
attenuating the secretion of or neutralizing the growth
suppressive effects of p53-dependent growth inhibitory
15 factors.

Having now generally described the invention, the
same will be more readily understood through reference to the
following example which is provided by way of illustration and
is not intended to be limiting of the present invention.
20

EXAMPLE

The present inventors proposed that p53 can act as a
tumor suppressor by inhibiting growth of damaged cells through
internal control of cell cycle and apoptotic machinery and
25 that the activation of p53 in normal cells induces the
production and release of growth inhibitory factors possessing
anti proliferative/anti-cancer activity. To test this
hypothesis, the growth inhibitory effect of conditioned media
from the cells differing in their p53 status before and after
30 p53 activation was compared. Three experimental cell systems,
representing pairs of isogenic cell lines differing in their p
53 status or activity, were specifically designed to analyze
p53 function as follows:

1)   p53-null human fibroblasts, strain MDAHO41
35 (Agarwal et al., 1995), originally obtained from a Li-Fraumeny
syndrome patient. MDAHO41 cells were compared with their
derivatives, TR9-7, obtained by introduction of wild type p53

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cDNA under the control of tetracycline-inducible promoter
(Agarwal et al., 1995). The level of p53 expression in TR9-7
in the presence of tetracycline is comparable to that of
normal p53-containing fibroblasts. p53 activation
5 (stabilization) in TR9-7 was achieved either by gamma
irradiation in the presence of tetracycline or by deprivation
of the antibiotic.

2) Balb 3T3 cells of cell line 12-1, express wild
type p53 (Harvey et al., 1991). This line was compared with
10 its variant obtained by retroviral transduction with anti-p53
GSE-56 acting as a strong dominant negative inhibitor of p53
function (Ossovskaya et al., 1996). p53 activation was
achieved by gamma irradiation.

3) Mouse embryo fibroblasts (MEF) with wild type
15 p53 transformed with a combination of Ela and transforming Ha-
ras (cell line C8) were compared with their derivatives
transduced with retroviral vectors expressing either anti-p53
genetic suppressor element (GSE) C2 (analog of GSE-56) or bcl-
2 (Nikiforov et al., 1997). The bcl-2-expressing construct
20 was used to prevent radiation-induced p53-dependent apoptosis
in C8 cells that may affect the properties of conditioned
media.

All the above cells were gamma irradiated (10 Gy) to
induce p53 in response to DNA damage, and conditioned media
25 was collected 8 hours after irradiation and compared with that
from control non-irradiated cells in a growth inhibitory
assay. In tet041 cells p53 was also activated with
tetracycline deprivation. A set of target prostate carcinoma
cell lines, ALVA, PC3, DU145, LNCAP, was used for growth
30 inhibition studies (Rokhlin et al., 1997). The effect of
conditioned media on growth of target cells was estimated by ^{3}H
thymidine incorporation after 12 hours of incubation in
conditioned media. As similar results were obtained for the
different target cell lines and, therefore, further analysis
35 was done using DU145 cells, known to be the most resistant
among the tested cell lines to conventional anti-cancer
treatments (low sensitivity to TGF-beta, interferon gamma, TNF

alpha, lack of apoptosis after treatment with adriamycin)
(Rokhlin et al., 1997).

In all three cell systems, growth inhibition of
target cells was evident only with the media from p53 wild
5   type cells after p53 activation. No such effect was detected
in the media from p53-negative cells regardless of whether
they were irradiated or not. Conditioned media from untreated
p53 wild type cells did not affect growth of target cells as
well. These observations indicate that cells can release
10   growth inhibitory factors in the media in response to stress-
treatments. It is important to note that irradiation of human
fibroblasts and Balb 3T3 cells did not lead to apoptosis
within the time of the experiment as judged by DNA degradation
assay. Moreover, bcl-2 does not prevent secretion of growth
15   inhibitory factors into media by C8 cells, although it blocks
apoptosis associated in C8 cells with p53 activation.

Secretion of growth inhibitory factors by cells with
wild type p53 in response to genotoxic stress (i.e., gamma
irradiation) raised the possibility that normal cells may
20   contribute to anti-tumor effect of radiation and chemotherapy
by releasing tumor-suppressive factors. This model provides a
possible explanation for well known phenomenon of "tumor bed
effect". To test this idea, multidrug resistant HeLa cells,
line KB8-5-11, overexpressing P-glycoprotein (P-gp) was used.
25   These cells are highly resistant to a variety of P-gp
substrates, many of which are used as chemotherapeutic drugs
(etoposide, adriamycin, Vinca alkaloids, etc.) (Roninson et
al., 1986). KB8-5-11 cells were plated (1000 cells per plate)
on sparse monolayers of either p53-deficient human fibroblasts
30   (MDHAO41) or their derivatives expressing wild type p53 (TR9-
7). When KB8-5-11 cells formed small colonies, 25mg/ml of
adriamycin, which is completely growth suppressive for human
fibroblasts (both lines) but does not affect growth of KB8-5-
11 cells, was added. The majority of adriamycin resistant
35   colonies on monolayer of cells with wild type p53 degraded
while similar colonies on p53-deficient cells continued to
grow. Morphological changes in dying cells indicated that

cell death occurred through activation of apoptosis. This
observation demonstrates that tumor cells can be killed by
chemotherapeutic drug indirectly through the p53-dependent
induction of growth suppressive factors in normal stromal
5 cells.

A similar experiment was done on colonies of
spontaneously transformed Balb 3T3 cells (line ConA carrying
lacZ gene under control of p53-responsive promoter, Roninson
et al., 1986) growing on human fibroblasts differing in their
10 p53 status (MDHAO41 and TR9-7). TR9-7 carry p53 cDNA under
the control of tetracycline-regulated promoter and activation
of p53 in them was achieved by tetracycline deprivation.

Several genes that are known to be p53-regulated
encode secreted growth inhibitory proteins. For example,
15 thrombospondin and IGFBP3 both are encoded by the genes that
are upregulated by p53 (Buckbinder et al., 1995; Dameron et
al., 1994). New candidate secreted growth inhibitors can be
found among other p53-responsive genes found by different
methods of identification of the genes whose expression
20 depends on p53 status. Thus, differential hybridization of
ATLAS cDNA arrays (CLONTECH Labs, Palo Alto, CA) with cDNA
probes obtained from gamma-irradiated p53-positive and
negative human fibroblasts and from irradiated spleens of p53-
wild type and p53-deficient mice revealed several new
25 candidate excreted growth suppressors including TGF-beta2,
inhibin-beta and a set of serine protease inhibitors.
Observations made by others were published suggesting the use
of serine protease inhibitor expression as potential
prognostic factors for the estimation of the efficacy of
30 radiation therapy and the risk of spontaneous cancer (Benitez-
Bribiesca et al, 1989 and Schelp et al., 1998). Chemical
inhibitors of serine protease have been used as anti-tumor
drugs for a number of years (DeClerck et al., 1994). These
facts clearly fit the present findings that link anti-cancer
35 effect of this class of anti-cancer secreted proteins with the
activity of p53 and anti-cancer therapy.

The obtained results indicate that anticancer

therapy can inhibit growth of tumor cells indirectly by
induction of p53-dependent secretion of growth inhibitory
factors by normal cells. Such factors can be found among p53
transactivators in culture media and presumably, in blood or
5 other fluids contacting with p53-wt cells in the organism
after anti-cancer treatment p53-dependent secretion of growth
inhibitory factors in response to stress is likely to explain
the mechanism of tumor bed effect of local gamma radiation on
proliferation of non-irradiated tissues. These factors could
10 be involved in control of aging since senescent cells
accumulated in aging tissues are known to activate p53-
dependent gene expression serving as a source of growth
inhibitors affecting proliferation and regeneration of
tissues.

15   The materials and methods used in the experiments in
this example are presented below along with a detailed
discussion of the results obtained.

MATERIALS AND METHODS
20 Cell lines
p53-negative Li-Fraumeni fibroblasts (line MDAHO41) their
derivative expressing wild type p53 under the control of a
tetracycline-regulated promoter (line TR9-7) and Balb/c 3T3
cells 12-1 (Harvey et al., 1991) with wild type p53 expressing
25 IacZ under control of p53-dependent promoter (line ConA) were
described earlier (Komarova et al., 1997; Agarwal et al.,
1995) and were kindly provided by George Stark. Mouse embryo
fibroblasts transformed with E1a+ras from p53-wild type (line
C8) and p53-null (line A4) fibroblasts were kindly provided by
30 (Lowe et al., 1993a). The derivatives of ConA and C8 cells
overexpressing bcl-2 or GSE-56 were obtained by retrovirus
transduction of the above cDNAs (Nikiforov et al., 1997).
Mouse breast epithelial cell line NMuMG and normal diploid
skin fibroblasts were obtained from ATCC. All cell lines were
35 cultivated in DMEM with 10% fetal calf serum. TR9-7 cells
were maintained continuously in the presence of 1 μg/ml of
tetracycline; to induce p53 expression, cells were incubated

without tetracycline for 24 hours. Prostate tumor cells ALVA,

PC3, DU145 and LNCAP were previously described (Rokhlin et
5 al., 1997). Short-term cultures of splenocytes and thymocytes
($2-3 \times 10^7$ cells/ml) were prepared from freshly isolated and
gently desegrated mouse spleens and thymus glands in RPMI
media supplemented with 10% fetal calf serum.

10 Animals
p53-knockout and control mice (both C57BL) were purchased from
Jackson labs, Bar Harbor, Maine.

Gamma-irradiation
15 Mice and cell cultures were irradiated (10 Gy) from a G.L.
Shepherd and Associated Model 143-68 Cs137 source at a dose
rate of 4 Gy per min.

cDNA array hybridization
20 Differential hybridization analysis was done with CLONTECH
ATLAS cDNA arrays (MacCallum et al., 1996) and with cDNA
microarrays provided by QBI Enterprises, Ltd. The ATLAS array
consists of 588 selected cDNAs on nylon membrane (MacCallum et
al., 1996). QBI arrays were prepared according to previously
25 described technology (Schena et al., 1995; website
www.clontech.com) by Synteni and contain approximately 20,000
cDNAs. Poly(A)$^+$ RNA was purified according to standard
protocols from different mouse tissues and human cell lines.
cDNA probe preparation and hybridization was done according to
30 published protocols and manufacturer's recommendations
(MacCallum et al., 1996; website www.clontech.com).

Preparation of media conditioned by gamma-irradiated human
splenocytes
35

Fresh human spleen was gamma irradiated (10Gy), desegregated
into a single-cell suspension in DMEM medium (5×10^7 cell/ml)

-19-

and incubated at 37°c for 7 hours. Cells were removed by
centrifugation and filtration of the supernatant through 0.45
μM filter. Conditioned medium was tested in growth inhibition
assay on DU145 cells.

5

Growth inhibition assay

Cell proliferation rate was estimated using ³H-thymidine
incorporation as previously describer (Rokhlin et al., 1997).
The experiments were repeated three times using four parallel
10 wells for each tested sample. Conditioned media from gamma-
irradiated and control cells and urine from irradiated and
control mice were collected 8 hours after irradiation, diluted
at different proportions with fresh media (5-50% for
conditioned media and 0.1-1% for urine) and loaded on target
15 cells for 48 hours. ³H-thymidine was added for the last 16
hours of incubation.

Tumor growth inhibition assay

10 athymic nude mice were injected subcutaneously with the
20 suspension of DU145 cells (0.1 ml of growth medium with 5x10⁵
cells per site, 4 sites per mouse). 5 mice were left
untreated while 5 mice were injected intravenously once a day
for 6 days with 0.15 ml of nondiluted conditioned media
prepared as described above. Mice were examined weekly for
25 tumor growth; the appearance of tumors detectable by palpation
is indicated in Figure 1.

Northern blot hybridization and semi-quantitative RT-PCR

The assays were performed according to standard and previously
30 described protocols in the references cited and incorporated
by reference herein.

Immunohistochemical detection of p53 and Western blot analysis
of p53 expression and in situ beta-galactosidase assays

35

These assays were performed as described (Komarova et al.,
1997).

Sequence analysis

DNA and mRNA sequences were retrieved from GENBANK Database
and searched for p53 binding sites within the range of ± 2 kb
relative to the trascription start point. One of two
5 additional mismatches and 1-7 bp gaps between half-sites were
allowed. Weakened consensus $RVNCHWGYYBN_{1-8}RVCWHGYYB$ was used
to allow for naturally occurring mutations in known p53
binding sites. Resulting sequences were accepted if they had
no more than 3-4 mismatches in total from the previously
10 defined consensus $RRRCWWGYYYN_{0-13} RRRCWWGYYY$ (El-Deiry et al.,
1992)and no more than 1 mismatch was allowed inside CWWG core
sequences per half-site dimer.

RESULTS

15 Identification of new p53-responsive genes

         The spectra of the p53-responsive genes that alter
their expression in response to gamma irradiation in a series
of cell systems (source of RNA for hybridization) differing in
their origin and p53 status were determined. Specifically,
20 p53-negative fibroblasts MDAH041 and its derivative, TR9-7,
which express tetracycline-regulated wild type p53 cDNA
(Agarwal et al., 1995) were used. The basal level of p53
expression in TR9-7 cells in the presence of tetracycline is
similar to that of normal human diploid fibroblasts;
25 accumulation of p53 in these cells is obtained by either gamma
irradiation or tetracycline deprivation. In addition to human
fibroblasts, mRNA from the spleen, thymus, and liver of wild
type or p53-knockout mice (Donehower, et al., 1992; Jacks et
al., 1994) either untreated or taken four hours after 10Gy of
30 gamma irradiation. Cells in the thymus and spleen undergo
massive p53-dependent apoptosis in response to DNA-damaging
treatments, while neither liver cells nor fibroblasts display
any evident fast response to DNA damage (Lowe et al., 1993c;
Komarova et al., 1997; MacCallum et al., 1996). ^{32}P-labeled
35 complex cDNA probes were synthesized for each cellular mRNA
populations and hybridized with the CLONTECH ATLAS cDNA arrays
(website www.clontech.com) (human or mouse, depending on the

mRNA origin) each containing 588 cDNAs. Fluorescently labeled cDNA probes from human fibroblasts were hybridized with two microarrays composed of about 20,000 known and unknown human cDNAs (Schena et al., 1995). Differential expression of the
5 genes detected by array hybridization was confirmed by Northern blot-hybridization and/or by semi-quantitative RT-PCR. The differential hybridization results obtained with the above set of cDNAs allowed the identification of mRNA species whose expression was affected by gamma irradiation, and
10 distinguished between p53-dependent and p53-independent genes. The results obtained are illustrated by Fig.2A and summarized in Tables 1 and 2.

Table 1: Mouse p53-responsive genes identified by hybridization to mouse cDNA array

#	Gene	Accession Number	Regulation			Putative p53 DNA-binding sites*
			spleen	Thymus	liver	
1	Cyclin G	Z37110	+	+	+	Okamoto et al., 1994
2	Cyclin B1	X64713	+	(-)	UD	nd
3	Waf-1	U09507	+	UD	UD	El-Deiry et al., 1993
4	p19ink4	U19597	+	NI	UD	nd
5	EI24	U41751	+	+	NI	>+470; >+871
6	Glutathione peroxidase	X84742	-(+)	NI	UD	NF
7	Glutathione S-transferase Pi	U15654	NI	NI	-(+)	-1249; +614
8	Glutathion S-transferase Mu1	J03914(R)	+	NI	NI	+167
9	Bax	L22472	+	+	UD	Miyashita et al., 1995
10	Alpha 1-proteinase inhibitor 2	X66454(R)	+	UD	NI**	-1153; +1489
11	Serine proteinase inhibitor 2	X16361	+	UD	+	-272
12	Serine proteinase inhibitor 2.4	X69832	+	UD	+(+)	>+566***
13	Nerve growth factor alpha	X01801	+(-)	UD	UD	NF
14	Neuroleukin	M14220	-(+)	NI	-(+)	>+642; >+834***
15	EGF	U69534	NI	NI	+	NF
16	Granulocyte-macrophage CSF receptor	M85078	+	UD	UD	NF***
17	Macrophage CSF-1 receptor (CSF-1)	U63963(H)	NI	NI	+	>+862; >+1540
18	Activating transcription factor 4	X61507	+	UD	UD	NF***
19	c-Jun protooncogene	U60582	UD	+	UD	NF
20	p45 NF-E2-related factor 2	U70474	+	NI	+	-608; -93
21	Cathepsin H	U06119	-(+)	NI	-(+)	>+986; >+1192
22	Alpha-actinin binding protein (Zyxin)	X99063	+	NI	UD	NF***

*position of putative p53 DNA-binding sites (distance from transcription start)
**very high expression level
***only coding sequence available
Abbreviations: + and -, p53-dependent induction and suppression; (+) and (-), p53-independent induction and suppression; NI, noninducible; UD, undetectable; NF, not found; nd, not done.
If no genomic sequences were available, closest homologs from other mammalian species were used: (R) - rat sequence; (H) - human sequence.

Table 2. Selected p53-responsive genes identified by hybridization to human cDNA arrays in human fibroblasts.

#	Gene	Accession #	Regulation	Putative p53 DNA-binding sites*
1	WAFl	U24171	+	El-Deiry et al., 1993
2	Glutathione peroxidase	M83094	+	-742; -211; +1548
3	Glutathione S-transferase mu	M96233 X68677	+	+529; +911
4	Inhibin-β A	U16239	+	+1484; +1743
5	TGF-β2	M87843	+	+1580
6	ID-1	U57645	+	+1538
7	ID-2	M97796	+	NF**
8	Ezrin	X51521	+	>+1791; >+1936**
9	Collagen α1 type IX	X54412	+	NF**
10	Collagen α1 type IV	X12784	+	-1095; +901; +1050
11	Collagen α1 type XIV	Y11709	+	>+871; >+584**
12	Collagen α1 type I	AF017178	+	+84; +595
13	Collage α2 type 1	AF004877	+	-919; +57
14	Microfibril-associated glycoprotein 2 (MAGP2)	U37283	+	NF**
15	Glia derived nexin-1	U33453	+	-1602; -1197
16	Antileukoproteinase 1	X04502	+	NF
17	Thrombospondin 2	W86006	-	Adolph et al., 1995
18	Interstitial collagenase	U78045	-	-569; +443
19	Hyaluronan synthase 2 (HAS2)	U54804	-	>+1596**
20	Stromelysin-1	U43511	-	-1579
21	Cathepsin O ≡ Cath K	U83273	-	>-762; >-501
22	Thrombomodulin	AA057068	-	Nd
23	Tissue factor pathway inhibitor-2	L27624	-	Nd
24	Plasminogen activator inhibitor-2, placental	AA032091	-	Nd
25	Prostaglandin G/H synthase	D28235	-	NF

*position of putative p53 DNA-binding sites (distance from transcription start)
**Only RNA sequences available
#1 - #8, genes identified using CLONTECH Atlas
#9 - #25, genes representing the strongest differentials among known genes in large (QBI) cDNA arrays

The genes identified include a number of known p53 responders (Tables 1 and 2): p21/*wafl*, *cyclin G*, *bax* and several genes encoding enzymes of glutathione metabolism. Only one gene, *cyclin G* (Okamoto et al., 1994), among those

5 tested, was recognized as a p53 responder in all mouse tissues analyzed. p53-dependent upregulation of p21/*wafl* and *bax* was evident in some of the cell types tested but was undetectable in others, possibly due to low basal expression levels. Transcription of the E124 gene (Lehar et al., 1996), was

10 activated by gamma irradiation in all mouse organs tested, but in the liver, this activation was p53 independent.

By comparing gamma-irradiated wild type p53 and p53-deficient human fibroblasts, several genes were found to be strongly repressed in the p53-wild type cells (nos. 18-25 in

15 Table 2 and Figs. 3A and 3B). Since in the experiments, mRNA was isolated shortly after the p53 activation, only those negatively regulated genes that were repressed prior to the p53 activation could reliably be detected. In fact, mRNA expression of such genes as stromelysin-1 (Figs. 3A and 3B),

20 Thrombomodulin or prostaglandin G/H synthase was deeply repressed in TR9-7 cells under normal conditions of growth (Table 2).

The largest group of p53 responders includes genes that are upregulated after gamma irradiation on p53 wild type

25 but not on p53-null background (*p21/wafl*, *cyclin G*, *TGF-β2*, etc). Another large group involves genes which basal expression levels was higher in p53-null cells than in p53 wild type cells. They were upregulated in p53 wild type cells and, in some cases, in p53-null cells after gamma irradiation

30 (*neuroleukin*, *ID-1*, *ID-3*, see Figs. 3A and 3B). Another group of genes are subjects of complex regulation (Figs. 2A and 2B). For example, cyclin B1 that is downregulated after gamma irradiation through the known mechanism involving mRNA degradation (Maity et al., 1995) was upregulated by p53

35 induced in TR9-7 cells by tetracyclin deprivation (Figs. 3A and 3B). The combined effects of the p53-dependent and p53-

-25-

independent mechanisms of cyclin B1 explains why the radiation
dependent decrease in cyclin B1 mRNA was more pronounced in
the p53-null than in the p53-wild type background (Figs. 3A
and 3B).

5   Interestingly, most of the arrayed genes that change
expression after gamma irradiation were found to be p53
dependent. For example, among 26 radiation-responsive genes
of the spleen, 19 were p53 dependent, confirming a unique role
of p53 in radiation response. Different cell types vary
10 dramatically in their content of p53-responsive genes. Only 8
of 19 p53-responsive genes in the spleen are found to respond
to p53 in other cell types analyzed. Remarkably, thymus and
spleen, which both are highly sensitive to p53-dependent
apoptosis, exhibit almost non-overlapping sets of p53-
15 dependent gene expression (Table 1). The tissue specificity
of p53 responsiveness of some of the identified genes seems to
simply reflect the differences in the basal levels of their
expression among tissues. For example, p53-induced
upregulation of *p19/ink4/arf* was evident in the spleen, where
20 its basal level is low, and could not be detected in the
thymus where its expression was high. *p19/ink4/arf* expression
in the liver was below the sensitivity of hybridization.
However, several genes (EGF, CSF-1, zycin, GST-μ1) with
similar basal levels of expression in different tissues showed
25 tissue specific response to p53, suggesting that p53 pathway
can be modulated by unknown tissue specific factors. Our
observation on tissue specificity of the majority of p53
responders suggests that some unknown factors target p53 to
different groups of promoters.
30

<u>p53-dependent cellular secretion of growth inhibitors *in vitro*</u>

          Newly identified p53 responders can be organized
into several groups according to their responses to p53 and
their functional activities. One of the major subgroups
35 includes several genes encoding enzymes of glutathione
metabolism, reflecting the recent discovery of p53 involvement
in the regulation of reactive oxygen (Hermeking et al., 1997).

Other subgroups involve transcription regulators (activating
transcription factor 4, c-Jun protooncogene, p45 NF-E2 related
factor 2 and ID-1, ID-2), growth factors and their receptors
(nerve growth factor alpha; neuroleukin; EGF; granulocyte-
5 macrophage CSR receptor; macrophage CSF-1 receptor(CSF-1)),
and extracellular matrix proteins (collagen α1 type IX;
collagen α1 type IV; collagen α1 type XIV; collagen α1 type I;
collagen α2 typeI). Some of the genes found are subjects of
complex regulation. For example, basal levels of neuroleukin
10 and cathepsin H mRNAs were significantly decreased in p53 wild
type cells and were upregulated after gamma irradiation in
p53-independent manner. Expression of serine proteinase
inhibitor 2 is induced by radiation in both p53 wild type and
p53-null splenocytes, but induction was significantly stronger
15 with p53 (Table 1, Fig.2A).

     Some genes that were upregulated in p53-dependent
manner after gamma irradiation in spleen, liver, and
fibroblasts encode secreted proteins with a known growth
inhibitory function. Different secreted inhibitors were
20 activated in different cell types. They include TGF-β2,
inhibin-β (in fibroblasts), and a variety of serine protease
inhibitors (*spleen, liver and fibroblasts*; alpha 1 -
proteinase inhibitor 2; serine proteinase inhibitor 2; serine
proteinase inhibitor 2,4 and glia derived nexin-1;
25 antileukoproteinase 1; thrombospondin 2). Insulin-like growth
factor-binding protein 3 (IGBP3) defined earlier as a p53
responder (Buckbinder et al., 1995) also belongs to this
group, although its p53-dependent upregulation was visible
only in human fibroblasts. The presence of a large number of
30 secreted inhibitors among p53-responsive genes suggests that
p53-dependent growth control may not be limited only to
suppression of damaged cell proliferation by internal
mechanisms, but may also involve induction and release of
growth inhibitory factors affecting the surrounding cells
35 (bystander effect). To test this hypothesis, the effect of
conditioned media from cells differing in their p53 status
before and after p53 activation was compared. As a source of

conditioned media, used several experimental cell systems, all
representing isogenic cell lines differing in p53 status or
activity was used. Specifically, (i) p53-null human
fibroblasts MDAH041 were compared with their derivatives TR9-
5 7, expressing tetracycline-regulated p53 (Agarwal et al.,
1995); (ii) ConA cells (Komarova et al., 1997) were compared
with their variant in which p53 function was suppressed by
transduction of a strong anti-p53 genetic suppressor element
GSE-56 (Ossovskaya et al., 1996); (iii) wild type mouse embryo
10 fibroblasts (MEF) transformed with an E1a and Ha-ras (line
C817; Lowe et al., 1993a) were compared with C8 cells
transduced with GSE-56 or bcl-2 and with similar fibroblasts
from p53-knockout mice; line at (Lowe et al., 1993a; Nikiforov
et al., 1997). All the above cells were gamma irradiated (10
15 Gy) to induce p53. Conditioned medium was collected 8 hours
after irradiation and compared with that from control non-
irradiated cells in a growth inhibition assays. Along with
gamma irradiation, tetracycline deprivation was used to
activate p53 in TR9-7 cells.
20   Several cell types were used as targets in growth-
inhibition assays. They include three prostate carcinoma cell
lines, PC3, DU145, and LNCAP and three nearly normal cells:
human and mouse diploid fibroblasts and nontransformed murine
mammary gland epithelial cells NmuMG. Similar results were
25 obtained with all cell lines, and we chose DU145 cells as
reference target cells for further analysis because they are
known to be the most resistant to conventional anticancer
treatments among the tested cell lines (Rokhlin et al., 1997).
          The results of ^{3}H-thymidine incorporation assays done
30 on DU145 cells are shown in Figs. 4A-4C. DU145 cells were
chosen for the demonstration of the effect since they are
resistant to many known negative regulatory stimuli, including
TGF-β, TNF-α, interferon-γ and FAS ligand; these cells are
p53-deficient and do not undergo apoptosis in response to
35 treatment with radiation or DNA-damaging chemo therapeutic
drugs (Rokhlin et al., 1997). Conditioned medium from
untreated p53 wild type cells did not affect the growth of

target cells. The medium from p53-negative cells had no
detectable growth inhibitory effects, regardless of whether or
not they were irradiated. In all three cell systems, growth
inhibition of target cells was evident only with the medium
5 collected from p53 wild type cells after p53 activation.
Growth inhibition was evident in all target cell types tested,
although the scale of the effect varied among different cells
(Fig. 5C). The strength of the growth inhibitory effect
correlated with the concentration of conditioned media and
10 could be enriched by ultrafiltration through Amicon filters
with the cutoff of 10kd (Fig. 5C).

It is important to note that radiation did not
induce apoptosis in human fibroblasts or ConA cells within the
time of the experiment, as judged by a DNA fragmentation assay
15 (data not shown), ruling out the possibility that the growth-
suppressive effect of the medium reflects cell degeneration.
Moreover, bcl-2 overexpression did not prevent C8 cells from
secreting growth inhibitory factors into the medium, although
it blocked apoptosis associated with p53 activation in C8
20 cells (Nikiforov et al., 1997) (Fig. 4A).

p53-dependent secretion of growth inhibitors ex vivo and in
vivo

25   Whether p53-dependent secretion of growth inhibitors
occurred in primary cell cultures and in vivo in the organism
in response to gamma irradiation was also assessed. Spleen
and thymus were chosen for these experiments because they both
respond to gamma irradiation by activation of massive p53-
30 dependent apoptosis but differ dramatically in contents of
p53-responsive genes (Table 1). Short-term cultures of
splenocytes and thymocytes from untreated and gamma-irradiated
p53-wild type and p53-null mice were prepared and the growth
inhibitory effect of conditioned medium at different times
35 after treatment was tested. Similarly, urine samples from
wild type and p53-knockout C57BL mice were analyzed before and

7 hours after being subjected to 10 Gy of gamma radiation.
The results of representative experiments summarized in Figs.
5A and 5B demonstrate the growth-suppressive effect detected
both *ex vivo* in conditioned media from splenocytes and *in vivo*
5 in urine of irradiated animals. In both cases, the effect was
p53-dependent (Figs. 5A and 5B). Conditioned media from
short-term cultures of irradiated thymocytes did not display
any detectable growth suppressive activity (data not shown);
consistently, no secreted proteins were found among the
10 products of a few p53-responsive genes of thymus (see Table
1). At the highest concentrations, the conditioned media from
irradiated splenocytes induced apoptosis in target tumor cells
(Fig. 1). Remarkably, the growth suppressive effects of
conditioned medium and urine were more pronounced in the three
15 tumor cell lines tested than in three types of normal cells,
including mouse and human diploid fibroblasts and mouse
mammary gland epithelial cells NmuMG (Fig. 5C).

p53-dependent bystander effect can potentiate the efficacy of
20 anti-cancer drugs

        The p53-dependent secretion of growth inhibitors
raises the possibility that chemo- and radiation therapy may
act not only by causing direct damage to tumor cells, but also
25 by inducing "export" of anti-proliferative stimuli and
the release of growth inhibitors by normal cells in response
to stress, thereby affecting growth of their neighbors. To
reveal this putative indirect action of chemotherapy, the
effect of a chemotherapeutic drug on drug-resistant tumor
30 cells, co-cultivated with nonmalignant fibroblasts, either
p53-deficient fibroblasts (MDAH041) or with their derivatives
expressing wild type p53 (TR9-7), was compared. To
distinguish between the direct and indirect effects of
chemotherapeutic drug treatment on tumor cells, the multidrug-
35 resistant tumor cell line KB8-5-11, which is resistant to
adriamycin due to the overexpression of P-glycoprotein (P-gp)
(Roninson et al., 1986), was used. KB8-5-11 cells were plated

on sparse monolayers of either p53-deficient (MDAHO41) or p53
wild type (TR9-7)human fibroblasts (1000 KB8-5-11 cells per
plate). Such an experimental design imitated tumor growth on
p53-deficient or p53-wild type stroma. Once fibroblasts

5   formed a complete monolayer and KB8-5-11 cells formed small
colonies, 50 ng/ml of adriamycin was added to the plates.
This drug concentration led to complete growth arrest (but not
apoptosis) in human fibroblasts (data not shown) and did not
affect growth of KB8-5-11 cells plated either alone or on the

10   layer of p53-deficient fibroblasts (Figs. 6A-6D). However,
most KB8-5-11 colonies growing on the monolayer of fibroblasts
with wild type p53 degraded, displaying morphological
characteristics of apoptosis (Figs. 6A-6D). This observation
demonstrates that the killing effect of chemotherapeutic drugs

15   on cancer cells can be determined in part by the p53 status of
surrounding normal cells - apparently through the p53-
dependent induction of secreted growth inhibitors. Similar
experiments were performed using spontaneously transformed
Balb 3T3 cells co-cultivated with on MDAHO41 and TR9-7 cells.

20   In these experiments, the induction of p53 expression was
achieved in TR9-7 cells by tetracycline deprivation. As in
the previous case, the growth of colonies of transformed cells
was significantly reduced on the TR9-7 background (data not
shown). These results demonstrate that activation of p53 in

25   normal cells leads to growth inhibition of co-cultivated tumor
cells apparently through bystander effect by surrounding
nonmalignant cells caused by the release of growth inhibitors.
This observation shows that the p53-dependent response of
normal tissues to anticancer treatments can facilitate the

30   efficacy of chemo- and radiation therapy.
        The p53-dependent secretion of growth inhibitors
with potential anticancer activity detected both in
conditioned medium and in body fluids indicates a novel growth
regulatory function of p53: stress-dependent cellular export

35   of growth inhibitory stimuli. This function possibly
determines the "bystander effect" associated with p53 gene
therapy (Qazilbash et al., 1997).

The list of identified growth inhibitors demonstrates their involvement in many well-known mechanisms of negative growth relation that have never before been associated with p53. Even a single group of p53-dependent
5 secreted proteins serine protease inhibitors, can inhibit cell proliferation through numerous signaling pathways (reviewed by Feinstein et al.). p53 can serve as a transcriptional suppressor (Mack et al., 1993), and several genes that are negatively regulated by p53 were found in this study (see
10 Tables 1 and 2). Some of the gene products of these genes have known functions that could contribute to the "exported" growth inhibitory effect of p53. Thus, deep suppression of hyaluronan synthase 2 (HAS2) is expected to cause a detrimental effect on cell proliferation by leading to a drop
15 in the synthesis of hyaluronan which is the involved in a variety of growth control pathways (Rooney et al., 1995). Remarkably, the observed growth suppression effect of p53-dependent secreted factors does not depend on the origin (mouse or human) and p53 status of the target cells: DU145 and
20 PC3 cells are p53-deficient, while the rest of the target cells tested have wild type p53.

Some of the p53-dependent factors have profound anticancer activity and are already used as supplements to chemotherapeutic treatments. These include members of the
25 TGF-β family and numerous serine protease inhibitors and their chemical functional analogs, which are emerging as potential tools to treat cancer (DeClerck et al., 1994).

The p53-dependent modulation of gene expression in gamma-irradiated fibroblasts (Table 2) clearly reflects
30 morphological and physiological alterations in irradiated tissues associated with growth attenuation of tumors transplanted in gamma irradiated organs (O'Brien et al., 1969) known as the tumor bed effort (TBE). Thus, induction of various collagen species and suppression of collagen-degrading
35 proteinases (stromelysin-1, interstitial collagenase and cathepsin K) correlates with deposition of collagen by connective tissue in coarse bundles following irradiation.

Deep p53-dependent suppression of HAS2 and COX2 genes,
determining production of potent angiogenic factors hyaluronan
and prostaglandin E (Rooney et al., 1995; Gullino et al.,
1995), along with the previously found activation of
5 thrombospondin 1 (Dameron et al., 1994), correlates with the
suppression of angiogenesis in irradiated tissues (O'Brien et
al., 1969). These observations indicate that TBE is a p53-
dependent phenomenon.

        So far, p53-dependent secretion of biologically
10 active factors was known to be involved in control of
angiogenesis (Dameron et al., 1994). The data show that this
mechanism of stress response involves numerous factors and
affects many different cell types, allowing one to revise the
role of p53 in the stress response *in vivo* and in anticancer
15 therapy by adding a new growth regulatory function associated
with this tumor suppressor. The expansion of this study to
other tissues and larger cDNA arrays is a straightforward way
to identify other p53-responsive secreted growth inhibitors
that would further our knowledge of the mechanisms of p53-
20 regulated stress response and lead to identification of new
biologically active molecules with potential therapeutic
applications.

        Morphological and physiological alterations of
normal tissues associated with aging may well be another
25 phenomenon related to the newly determined function of p53.
Senescent cells that accumulate in tissues over time (Dimri et
al., 1995) are known to maintain very high levels of p53-
dependent transcription (Atadja et al., 1995). p53-dependent
secretion of growth inhibitors by senescent cells,
30 accumulating in aging tissue, may affect proliferating cells
and lead to a gradual decrease in overall proliferation
capacity of tissues associated with age. Identification of
p53-dependent growth inhibitors allows this hypothesis to be
tested directly.

35   Having now fully described this invention, it will
be appreciated by those skilled in the art that the same can
be performed within a wide range of equivalent parameters,

concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in
5 connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from
10 the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal
15 articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the
20 entire contents of the references cited within the references cited herein are also entirely incorporated by references.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or
25 embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the
30 skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and
35 modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be

understood that the phraseology or terminology herein is for
the purpose of description and not of limitation, such that
the terminology or phraseology of the present specification is
to be interpreted by the skilled artisan in light of the
5 teachings and guidance presented herein, in combination with
the knowledge of one of ordinary skill in the art.

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What Is Claimed Is:

1. A method for preparing p53-dependent growth inhibitory factors, comprising the steps of:

culturing human cells capable of expressing p53 tumor suppressor protein in a nutrient medium;

inducing expression and secretion of p53-dependent growth inhibitory factors by the cultured human cells into the nutrient medium to form a conditioned medium; and

recovering the p53-dependent growth inhibitory factors from the conditioned medium to provide a preparation of the p53-dependent growth inhibitory factors.

2. The method according to claim 1, wherein the nutrient medium for culturing human cells is a serum-free medium.

3. The method according to claim 1, wherein the cultured human cells are fibroblasts.

4. The method according to claim 1, wherein the cultured human cells are a mixture of cell types capable of expressing p53 tumor suppressor protein and capable of being induced to express and secrete p53-dependent growth inhibitory factors.

5. The method according to claim 1, wherein the p53-dependent growth inhibitory factors expressed and secreted by the cultured human cells are selected from at least one of the group consisting of TGF-beta 2, inhibin-beta, and serine protease inhibitors.

6. A pharmaceutical composition for reducing unwanted or abnormal cell proliferation, comprising a preparation of p53-dependent growth inhibitory factors prepared according to claim 1.

7. The pharmaceutical composition according to claim 6, further comprising a pharmaceutically acceptable carrier, excipient, diluent or auxiliary agent.

8. A method for reducing unwanted or abnormal cell proliferation, comprising the step of administering an effective amount of the pharmaceutical composition according

to claim 6 to a subject in need thereof.

9.   The method according to claim 8, wherein the cell proliferation is cancer.

10.   The method according to claim 9, further comprising administering a chemotherapeutic compound to the subject or irradiating the subject with gamma radiation.

11.   A method for preventing or delaying cell senescence, comprising the step of administering to a subject in need thereof an effective amount of a pharmaceutical composition comprising an agent which attenuates the secretion of or neutralizes the growth suppressive effects of one or more p53-dependent growth inhibitory factors.

FIG. 1

—+— CONTROL —△— TREATED
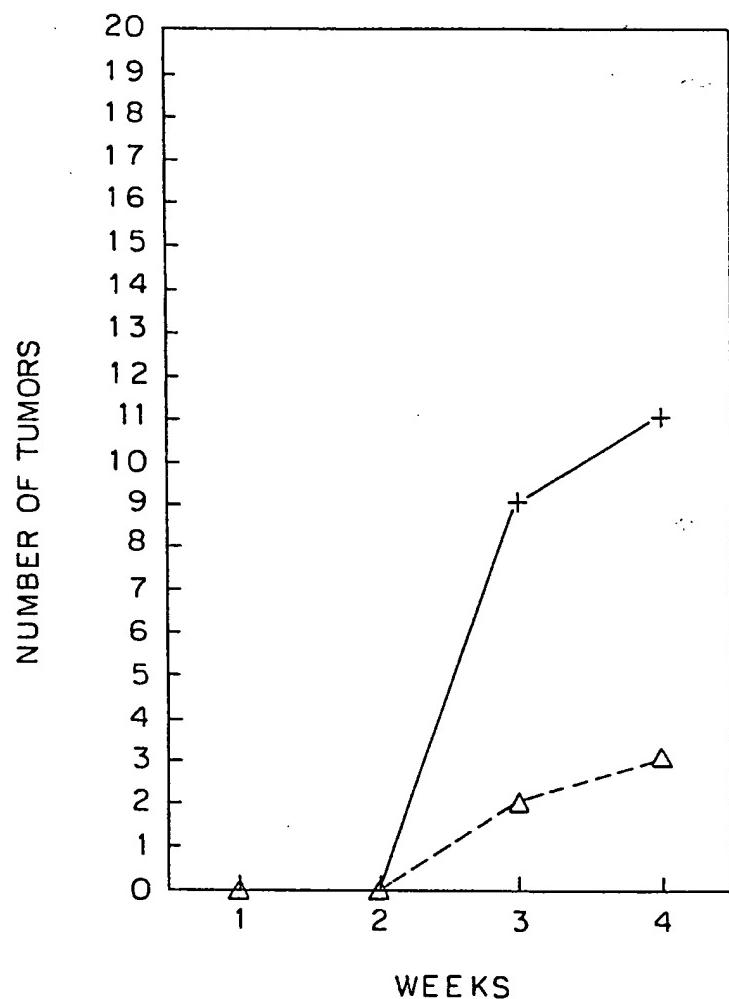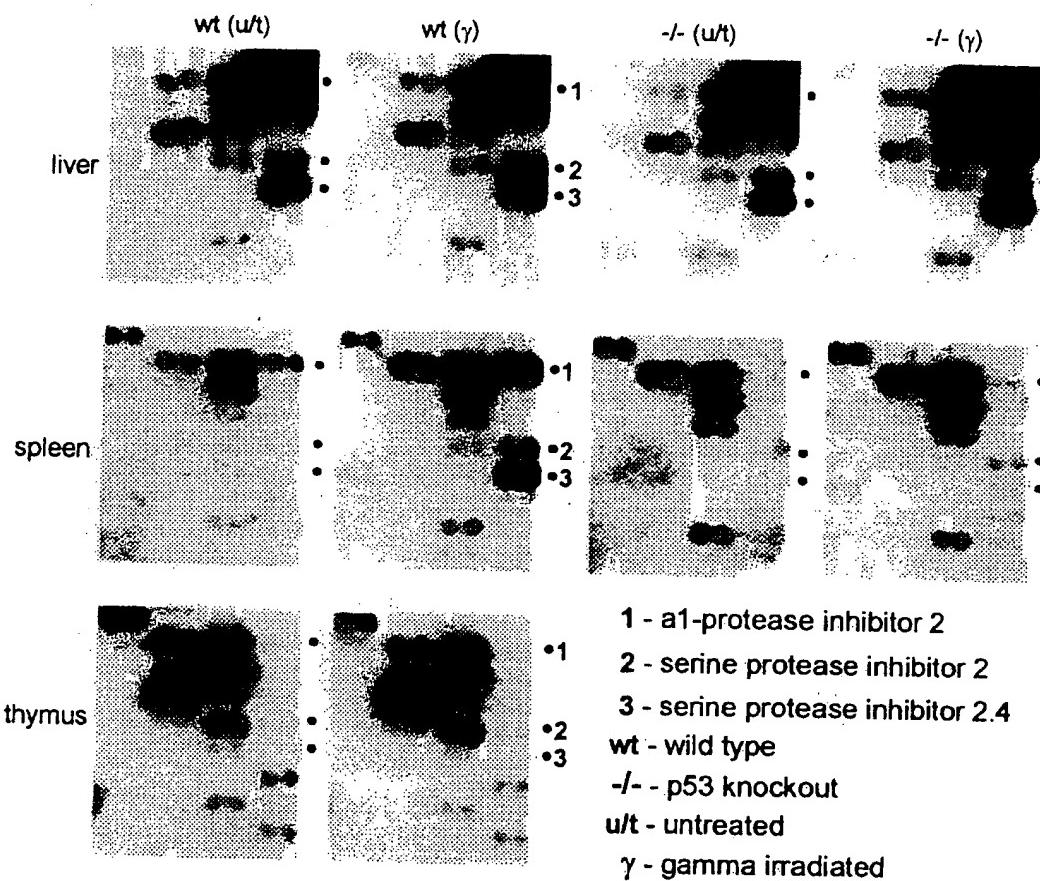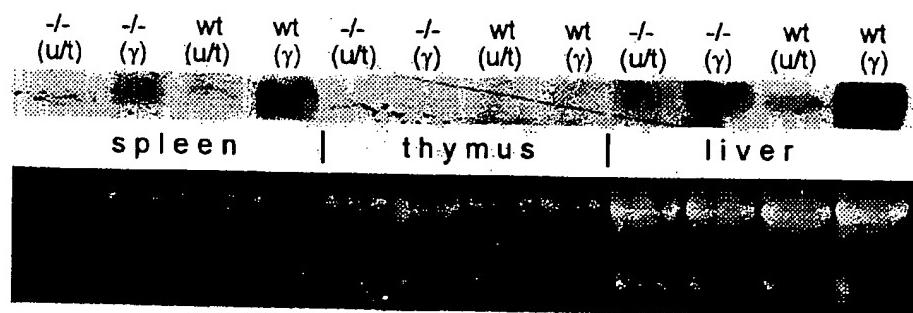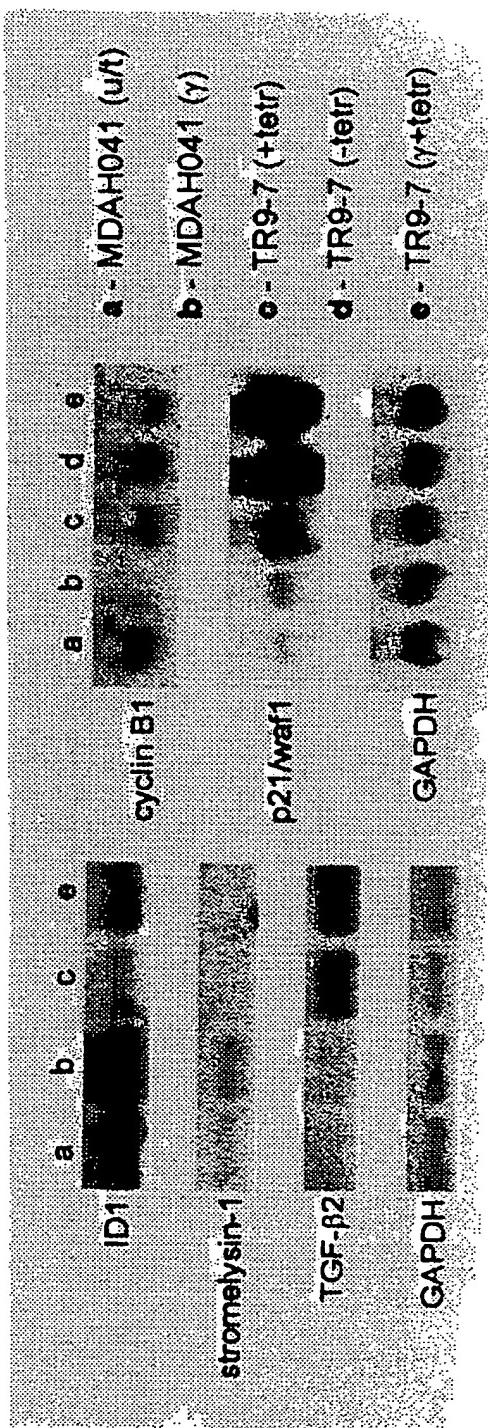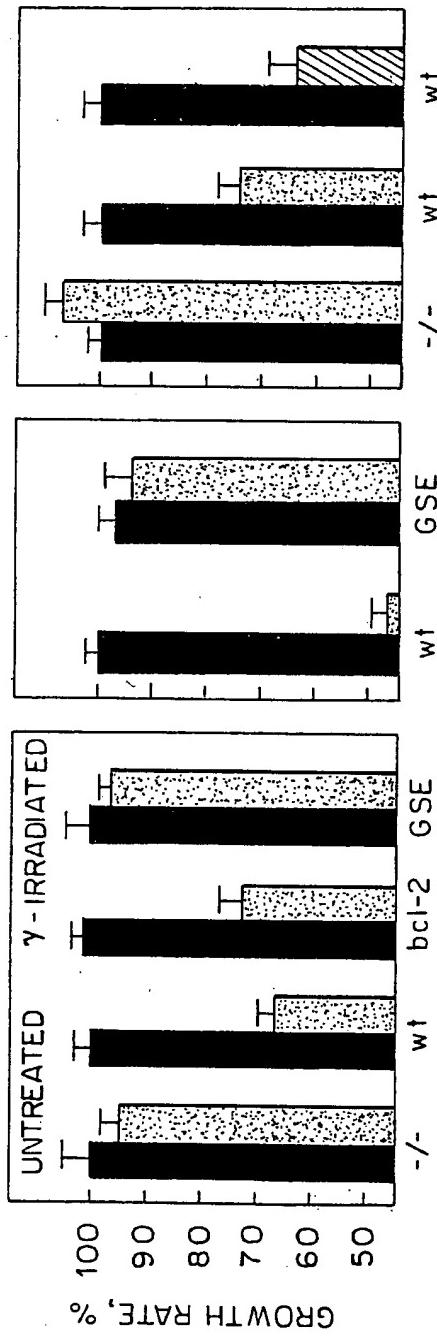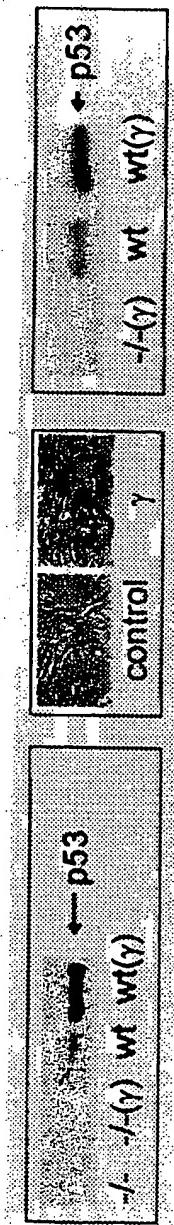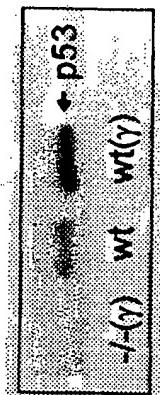
FIG. 2A

	wt (u/t)	wt (γ)	-/- (u/t)	-/- (γ)

liver

spleen

thymus

1 - a1-protease inhibitor 2
2 - serine protease inhibitor 2
3 - serine protease inhibitor 2.4
wt - wild type
-/- - p53 knockout
u/t - untreated
γ - gamma irradiated

FIG. 2B

-/- (u/t)	-/- (γ)	wt (u/t)	wt (γ)	-/- (u/t)	-/- (γ)	wt (u/t)	wt (γ)	-/- (u/t)	-/- (γ)	wt (u/t)	wt (γ)

s p l e e n | t h y m u s | l i v e r

FIG. 3B

FIG. 3A

FIG.4C

FIG.4B

FIG.4A

FIG.4F

FIG.4E

FIG.4D

FIG.6A

FIG.6B
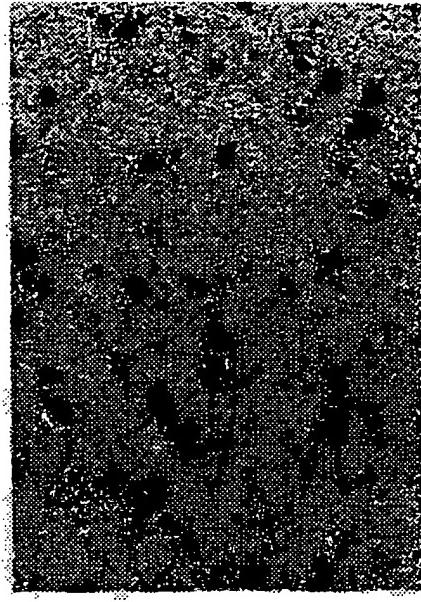
FIG.6C
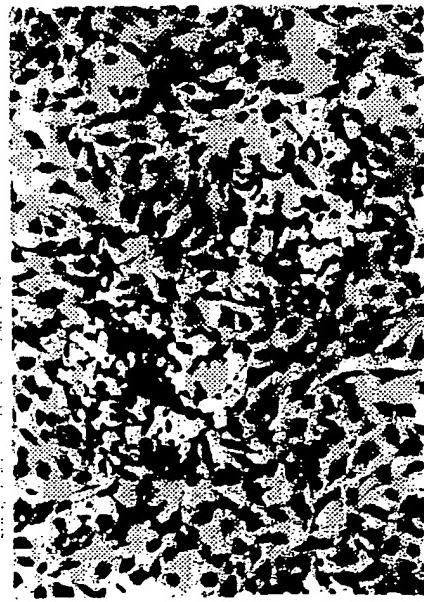
FIG.6D

FIG.5A
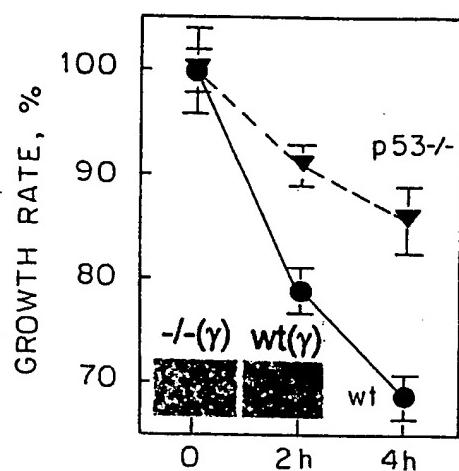
FIG.5B
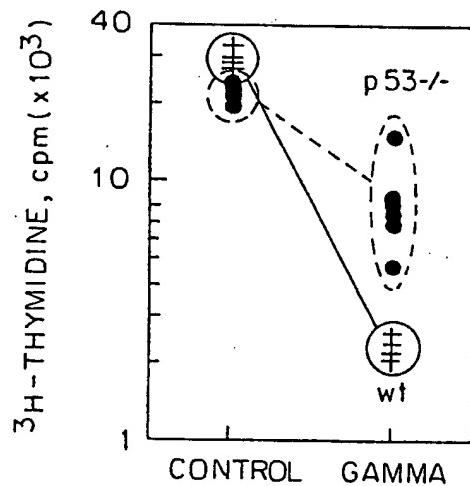
FIG.5C
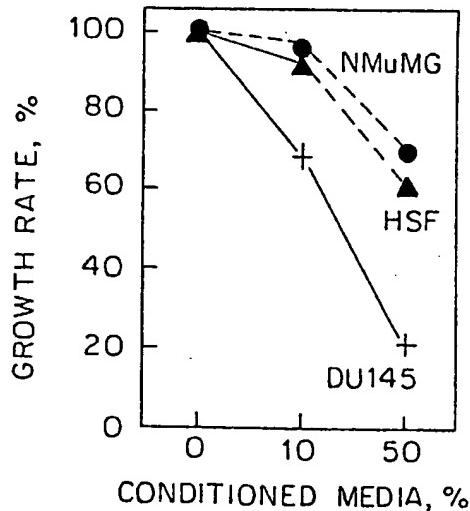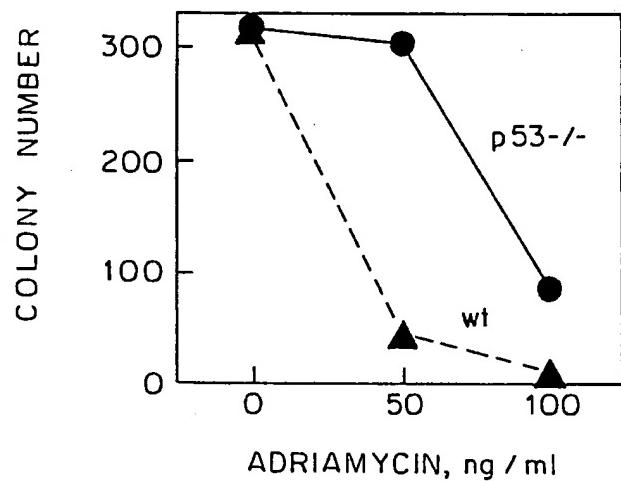
FIG.7

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 37/18; C12N 5/06; A61K 38/60; C07K 1/00
US CL : 514/2; 435/325; 530/344, 361

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2; 435/325; 530/344, 361

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Database SCISEARCH on STN. TANG et al. 'Expression of Transforming Growth-Factor-Beta (TGF-BETA) Isoforms and TGF-Beta Type II Receptor Messenger-Ribonucleic-Acid and protein, and the Effect of TGF-Beta-S on Endometrial Stromal Cell-Growth and Protein Degradation In-Vitro'. Endocrinology. July 1994, Vol. 135, No. 1, pages 450-459, see Abstract.	1,4,5,6 ------- 2,3,7
X -- Y	Database CAPLUS on STN. BALE et al. 'Regulation of Insulin-Like Growth Factor Binding Protein-3 Messenger Ribonucleic Acid expression by Insulin-Like Growth Factor I'. Endocrinology. 1992, Vol 131, No. 2, pages 608-614, see Abstract.	1,3,6 ----- 2,7
A,P	Database EMBASE on STN. KOMAROVA et al. 'Stress-Induced Secretion of Growth Inhibitors: A Novel Tumor Suppressor Function of p53'. Oncogene. 03 September 1998, Vol. 17, No. 9, pages 1089-1096, see Abstract	1-7

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 MAY 1999	28 MAY 1999

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JOYCE BRIDGERS PARALEGAL SPECIALIST SUSAN UNGAR CHEMICAL MATRIX
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/03777

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

> Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)⋆

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS, CANCERLIT, CAPLUS, DDFU, DRUGU, EMBASE, MEDLINE, SCISEARCH, TOXLINE, TOXLIT, USPATFULL
search terms:culture, secrete, fibroblast, human, tgf-beta 2, cancer, tumor, treat therapy, senescence, neutralize

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s)1-10, drawn to a pharmaceutical composition for reducing unwanted or abnormal cell proliferation, that is a p53 growth inhibitory factor, a method of making and a method of using said composition.

Group II, claim(s) 11, drawn to a method of preventing or delaying cell senescence comprising an inhibitor of the composition of Group I.

The inventions listed as Groups do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I is considered to be a p53 growth inhibitory factor.

The special technical feature of Group II is considered to be an inhibitor of p53 growth inhibitory factor.

Accordingly, Groups I-II are not so linked by the same or a corresponding special technical feature as to form a single general inventive concept.